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Research Article

Antimicrobial, Antioxidant and Anticancer Activities of Gold Nanoparticles Synthesized Using *Boswellia serrata* Aqueous Seed Extract

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ABSTRACT

In this paper present study gold nanoparticles (AuNPs) were synthesised using seed extract of *Boswellia serrata* aqueous seed extracted which is considered as waste material and generally thrown away into the environment. The bioactive molecules in the seed act as reducing agent to synthesise AuNPs without using any external agent. The characterisation of green synthesised gold nanoparticles was done using various spectroscopic techniques. Visual colour change from colour less to ruby red colour confirmed the formation of AuNPs which was further confirmed by optimal density peak at 537 nm by UV-spectra. FTIR analysis confirmed the presence of alcohol, phenol, carboxylic acid, ketones, amines, aromatic amines, aliphatic amines, alkyl halides and alkynes in *Boswellia serrata* seed which were responsible for the reduction of gold to gold nanoparticles. The green synthesised gold nanoparticles (AuNPs) were evaluated for their antimicrobial, antioxidant and cytotoxic potential. Seeds of *boswellia serrata* instead of discarding can be successfully utilised for AuNPs synthesis.

1. Introduction

Fruits are generally thrown into the environment as waste material causing pollution and their disposal is a major problem, but these parts like any other part of the plant are endowed with phytoconstituents which can be therapeutically exploited. Fruit and vegetable peels as a source of antimicrobics are reported by various researchers [2,3]. The seeds and peels show many promising biological activities like antimicrobial, antioxidant, anti-cancer, anti-inflammatory, wound healing, anti-ulcer, etc [4] and thus can be used for pharmaceutical purposes. All parts of the plant like leaves, bark, fruit peel and flesh, roots, and flowers have many medicinal properties and are traditionally used to treat many diseases [5]. Infectious diseases and cancer are two major health hazards globally and are one of the leading causes of death. Cancer too has become a deadly dangerous disease, Treatment options are few and many anti-cancer drugs in use are of natural origin and the synthetic drugs in use are becoming less popular because of their many side effects, costly and increase in chances of reoccurrence [6]. It has revolutionized the new era. The nanoparticles show entirely different properties than the bulk materials from which they are synthesized. Metal nanoparticles like silver, gold, zinc, copper, palladium, etc can be synthesised by various physical, chemical and biological methods. Biological methods make use of different parts of the plant like leaf, stem, peel, seed, flower, fruit and root, etc. These parts are rich in a variety of phytoconstituents which act as reducing and stabilizing agent in nanoparticles formation. There is no need of adding any other reducing or stabilizing agent. The medicinal plants themselves are a natural source and act as antimicrobics, antioxidant, anticancer,etc agents and nanoparticles synthesized using them is all the more exhilarating since nanoparticles because of their smaller size, large surface to volume ratio show more promising activity. The green synthesis is simple, easy, cost-effective, rapid and ecofriendly [7]. There are many applications of AuNPs viz. drug delivery [8] antimicrobial [9], anti-cancer [10], antioxidant and catalytic activities [11] etc. In this work, we report perhaps for the first time, synthesis of gold nanoparticles from seed extract of Boswellia Serrata. Characterization was done using various spectroscopic techniques like UV-Vis, FTIR, DLS and Zeta potential. Further anti-microbial, antioxidant and three different anticancer activities were evaluated.

2. Materials and Methods

2.1 Plant Material

The fruits of seed *Boswellia serrata* were collected from local area, Warangal region, Telangana state, India. The fruits seed

Figure 1. Colour change in the reaction mixture indicate formation of AuNPs.

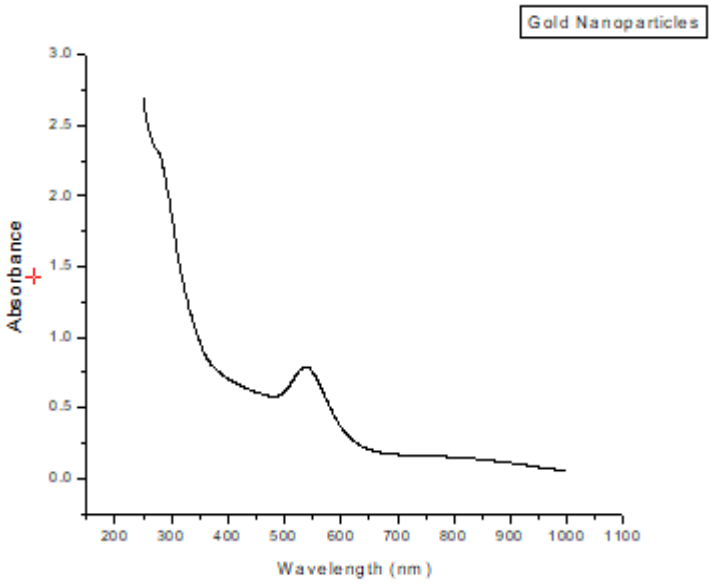

Figure 2. UV-Visible spectrum of AuNPs

were separated and washed thoroughly with tap water and the seeds were manually separated after breaking fruit seed, shade dried at room temperature and homogenized to fine powder prepared.

2.2 Preparation of Seed Extract

Boswellia serrata seed extract was prepared by decoction extraction method. One gram dry powder was extracted with100 ml of distilled water by boiling for 5 min. The extract was filtered through What man filter paper No. 1 and centrifuged at 5,000 rpm for 20 minutes. The filtrate was used for the synthesis of AuNPs.

2.3 Synthesis of AuNPs

Aqueous solution of Gold (III) chloride trihydrate ($HAuCl_4$) (1 mM) was prepared and used for the synthesis of AuNPs. About 6 ml of freshly prepared seed extract was added to 40 ml of 1 mM $HAuCl_4$ solution at room temperature (25 °C ± 2 °C) and incubated in dark for 24 h. The nanoparticles solution was purified by repeated centrifugation at 5,000 rpm for 10 min followed by redispersion of the pellet of nanoparticles. After air drying, the purified nanoparticles were stored at 4 °C for analysis.

2.4 Characterization of the Synthesized AuNPs

2.4.1 UV-visible Spectroscopy

The formation of AuNPs was determined using a UV–Vis spectrophotometer (Shimadzu UV-1601) in 350–750 nm range operated at a resolution of 10 nm. Base line correction of the spectrophotometer was performed using a blank reference. The reduction of the Au+ ions in solution was monitored by periodic sampling of aqueous component and measuring the UV-Vis spectra of the solution.

2.4.2 Dynamic Light Scattering (DLS), Zeta Potential (ZP) and TEM Imaging

AuNPs were analyzed in DTS0012 sizing cuvette with an optical path length of 1 cm at 25 °C for DLS. 630 µl of AuNP sample was analyzed using DTS1060C Clear dispensable zeta cell at 25 °C for zeta potential. Measurement of the size (DLS) and zeta potential (surface charge) of gold colloidal particles was carried out using Zetasizer Nano Series (Nao-ZS), Malvern Instruments Ltd. (Malvern, UK).

2.4.3 Fourier Transform infrared Spectroscopy (FTIR) Analysis

Possible functional groups involved in the synthesis and stabilization of AuNPs was determined by FTIR spectroscopy. The FTIR spectrum was recorded in the range of 400–4000 cm-1. Various modes of vibrations were identified and assigned to determine the different functional groups present in *boswellia serrata* seed extract and nanoparticles.

2.5 Antimicrobial Activity

The antimicrobial activity was evaluated against *E.coli, Pseudomonas putida, Staphylococcus aureus* and *Micrococcus luteus* cultures was taken from Department of Biochemistry, Osmania University, Hyderabad.

2.6 Agar Well Diffusion Assay

In vitro antimicrobial activity was determined by agar well diffusion assay [12]. Five different concentrations (20 µg/20 µl, 40 µg/40 µl, 60 µg /60µl , 80 µg/80 µl and 100 µg/100µl) of AuNPs were made in 100% distilled water, 100microlitters of different concentrations of nanoparticles was added into the well. Distil water was used as a negative control. Antimicrobial activity was assayed by measuring the diameter of the zone of inhibition formed around the well (mm). The experiment was done in triplicate, and the average values were calculated for antimicrobial activity.

2.7 DPPH Radical Scavenging Activity

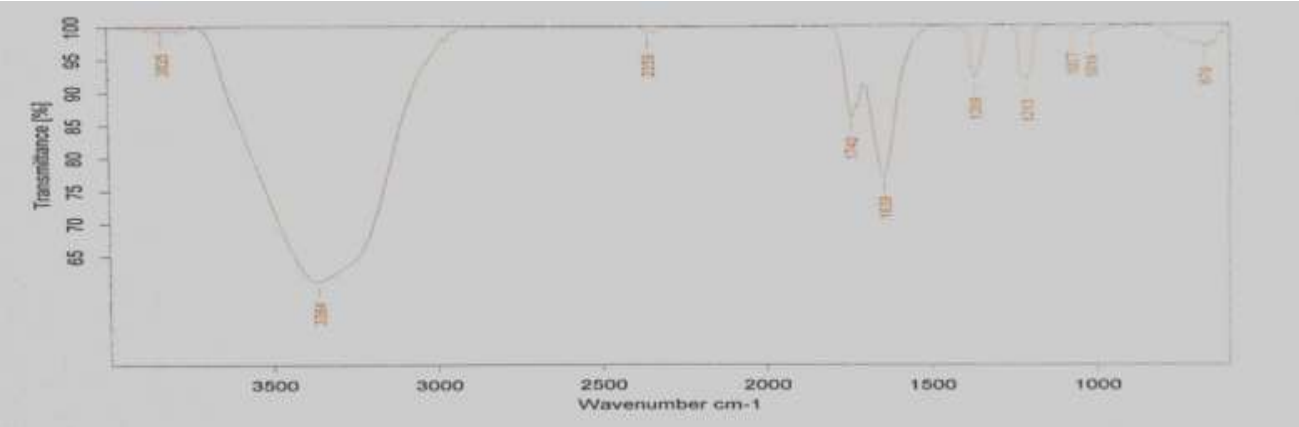

Figure 3. Fourier Transform Infra-red spectroscopy (FTIR) spectrum of AuNPs

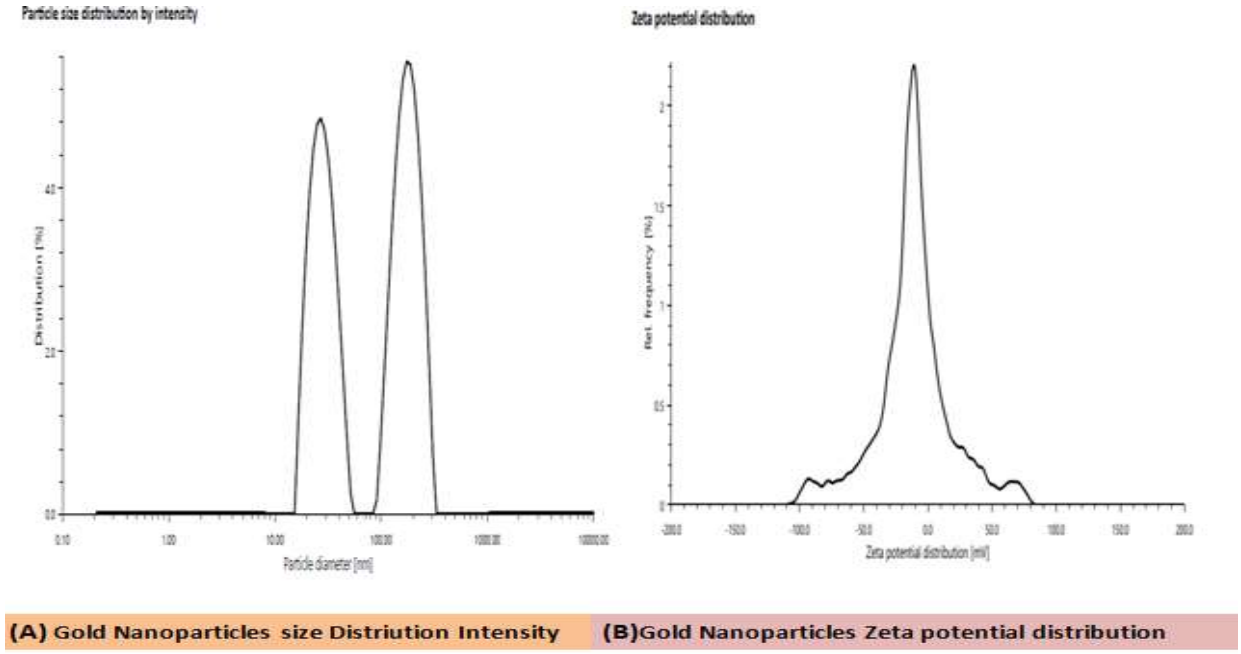

Figure.4. Gold Particle size distribution and Zeta potential graphs of synthesized from *Boswellia serrata* aqueous seed extract.

2.7.1 Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical Scavenging Assay

The biosynthesized AuNPs and the aqueous seed extract were tested for their antioxidant activity by DPPH method . Each extract (0.2, 0.4, 0.6, 0.8 or 1 mg/mL) was mixed with 3 mL of methanolic solution containing DPPH radicals (0.1 mM). After 30 min, absorbance was determined at 517 nm. The percent inhibition of activity was calculated as [(Ao - Ae)/Ao] * 100 (Ao = absorbance without extract; Ae = absorbance with extract). The results were expressed as IC50 which is the concentration of the sample required to inhibit 50 % of DPPH concentration.

2.8 In Vitro Cytotoxic Activity

Cancer cell viability of the AuNPs was evaluated by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay [13]. The cell lines uses were Human cervical cancer cell line (HeLa), Breast Cancer cell line (MCF-7) and Fibroblast normal cell line. The cell lines were obtained from National Repository of Animal Cell Culture, National Centre for Cell Sciences (NCCS), Pune, India.

3. Results and Discussion

Synthesis of AuNPs using *boswellia serrata* extract. Synthesis of nanoparticles was visually seen by a colour change in reaction mixture indicating the formation of nanoparticles by *Boswellia serrata* seed extract. Green synthesis of AuNPs, involved the addition of *Boswellia serrata* seed extract to gold chloride trihydrate solution, the synthesis reaction started with in few minutes and colour change occurred in the reaction mixture. The formation of AuNPs was preliminarily confirmed by a colour change from colour less to purple red colour solution within 5 min indicating the formation of AuNPs. As the incubation time increased, colour intensity also increased because of more synthesis of AuNPs and finally it turned to dark purple red colour at 24 h (Figure 1**).** Such visual colour change indicated the formation of AuNPs which is due to surface Plasmon resonance which is a collective excitation and oscillation of the surface electrons present in the surface conduction band of metal nanoparticles. Conversion of colour less reaction mixture to cherry red colour on addition of *Boswellia serrata* leaf extract indicated the formation of AuNPs [14].

Table.1 Effect of green synthesized AuNPs against on gram positive and gram negative zone of inhibition in (mm).

Antibacterial activity of gold nanoparticles						
Strain Names	20µg/20µl	40µg/40µl	60µg/60µl	80µg/80µl	100µg/100µl	Ampicillin (50µg/50µl)
E.coli	3.2	5.7	8.2	11.6	14.2	15.4
Pseudomonas putida	4	6.4	9.7	13.4	13.9	15
Staphylococcus aureus	4.2	6	8.6	12.8	15	16
Micrococcus luteus	4.3	5.8	8.4	12.7	16.4	17

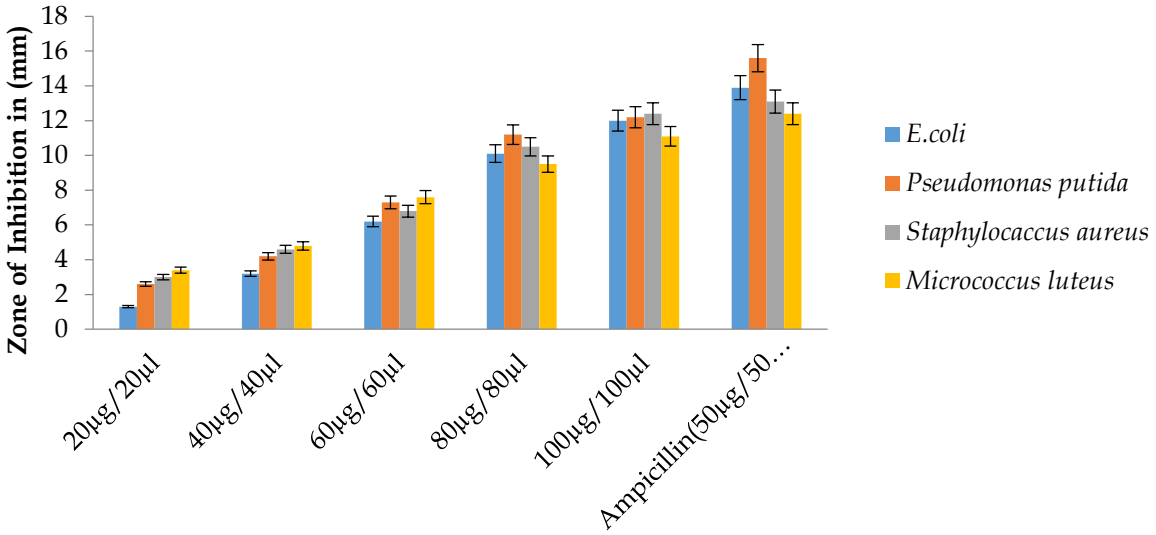

Figure 5. Zone of Inhibition (mm) Antibacterial activity of gold nanoparticles

3.1 Characterizations of AuNPs

3.1.1 UV-Vis Spectrophotometer Analysis

Boswellia serrata seed extract mediated synthesis of AuNPs was confirmed by UV-Vis spectroscopic studies. The absorption spectrum of synthesized AuNPs was found to have amaximum absorption band in the range of 200–1100 nm. Which is due to the SPR (Surface plasmon resonance) of AuNPs with a maximum absorption peak at 537 nm (0.7897 O.D Value) (Figure.2). This is characteristic nature of noble metal gold and several other researchers showed an absorption peak at 530 and 560 [15,16].

3.2 Fourier transforms Infrared Spectroscopy (FTIR)

FTIR spectra of AuNPs showed prominent peaks at 3835 cm^{-1}, 3364 cm^{-1}, 2359 cm^{-1}, 1740 cm^{-1}, 1639 cm^{-1}, 1369 cm^{-1}, 1213cm^{-1}, 1077 cm^{-1},1019cm^{-1}, 679 cm^{-1} **(Figure.3).** The absorption band at 3364cm^{-1} corresponds to O–H medium stretching of alcohol group. The absorption band at 2359 cm^{-1}corresponds to C=C- medium stretching of alkyne group. The absorption band at1740 cm^{-1}corresponds to C=O broad stretching of acid group. The absorption band at 1639 cm-1 corresponds to C=C medium stretching of non-conjugated diene group. The absorption band at 1369 cm^{-1} corresponds to C–F medium stretching of alkyl halide group. The absorption band at 1213 cm^{-1}corresponds to C–N strong stretching of amine group. The absorption band at 1369 and 1213 cm^{-1} corresponds to C–N broad stretching of amine group. The absorption band at 1077 cm^{-1} corresponds to C–F broad stretching of alkyl halide group. The absorption band at 1019 and 871.85 cm^{-1} corresponds to =C–H broad bending of alkene group. The absorption band at 679 cm^{-1} corresponds to =C–H medium bending of alkene group.

Plant extracts contain different secondary metabolites which were responsible for synthesis, reducing and stabilizing the AuNP formation. Barai et al. [17] attributed the plant secondary metabolites such as polyphenols in cluding flavonoids, steroids, etc for green synthesized AuNPs from stem bark extract of Nerium oleander [18] synthesized AuNPs using Cannabis sativa which exhibited antibacterial, antifungal, anti-inflammatory and anticancer properties and the some bioactive molecules like flavonoids, cannabinoids, phenols and terpenes present in the plant were responsible for reducing arnium chloride.

3.3 Zeta Potential Analysis

The particle size distribution and Zeta potential graphs of AuNPs are given in (Figure 4). The Zeta potential of green synthesized AuNPs was-24.86 mV. The charge of the Zeta potential indicates the stability of nanoparticles. Higher the negative value of Zeta potential more is the inter particle repulsion and more is the stability. In this study, the green synthesized gold nanoparticles showed moderate stability

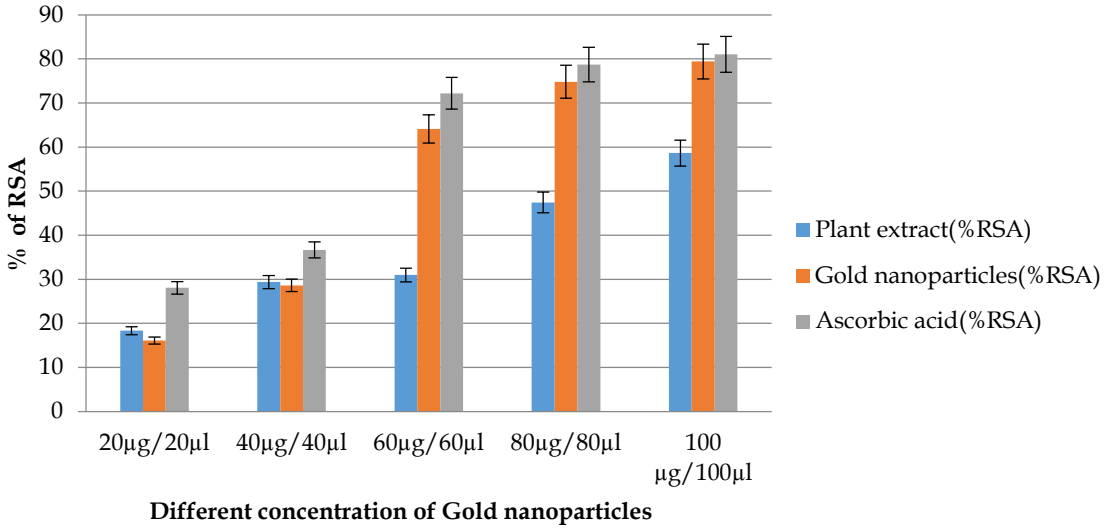

Figure 6. Antioxidant activity of AuNPs: DPPH free radical scavenging activity

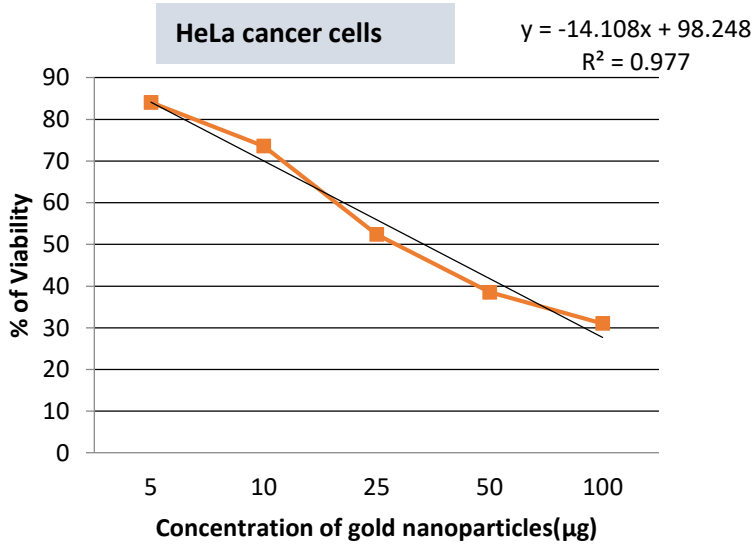

Figure-7 % of viability analysis of *Boswellia serrata* seed extract AuNPs on HeLa cancer cell line Affected drastically even at a high concentration

3.4 Antimicrobial Activity

The antibacterial activity of green synthesized AuNPs against Gram-positive and Gram-negative**.** The antibacterial activity was more towards Gram-positive bacteria than Gram-negative bacteria. AuNPs could inhibit almost all the four Gram-positive bacateria at three higher concentrations (Table 1 and Figure 5). AuNPs inhibited all the four Gram-negative bacteria at two higher concentrations. All the four clinical isolates were inhibited at two higher concentrations. On the whole, the antibacterial activity was more antibacterial activity towards Gram-positive bacteria was more than that against Gram-negative bacteria. This differential activity is attributed to the different cell wall make-up of Gram-positive and Gram-negative bacteria. The cell wall of Gram-positive bacteria is made up of thick peptidoglycan layer while that of Gram-negative bacteria is thin and made up of lipo polysaccharides which is impermeable to lipophilic substances. Though the exact mechanism of antibacterial action is not very well understood it is suggested that nanoparticles induce morphological changes in cell wall membrane structure there by disrupt the membrane permeability, disrupt the respiratory function ultimately leading to cell death. They induce the formation of irregularpitson the cell wall interact with proteins forming complexes with S, N, P and oxygen atoms and cause irreversible damages in the cell wall. Some Free radicals in nature like OH, SO, HOCL, H2O2, NO, RCOO are also formed which interact with bimolecules like lipids, proteins, enzymes and DNA. Some of the damagescaused in DNA are mutations, additions, deletions, single-breaks, double-strand breaks and cross-linking with proteins [19].

3.5 Antioxidant Activity

The DPPH radical scavenging activity of green synthesized AuNPs at different concentrations is given in (Figure 6**).** All showed dose-dependent antioxidant activities based on the concentration of nanoparticles increased, % inhibition also increased. In the concentration range of 80–480 mg/ml, an inhibition of 35–96% was envisaged for green synthesized AuNPs and IC_{50} value was 256 mg/ml for DPPH activity. Similar results of AuNPs synthesized using roots of Angelica

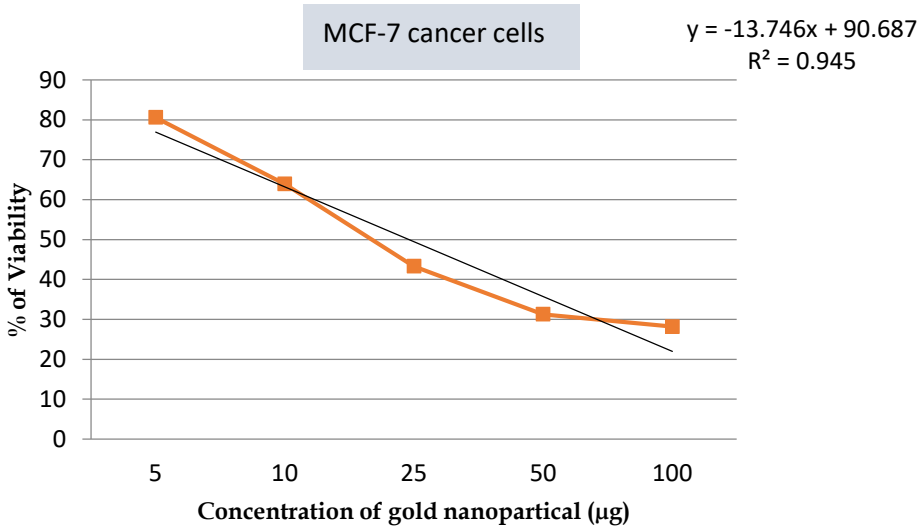

Figure-8 % of viability analysis of *Boswellia serrata* seed extract AuNPs on MCF-7 Cells.

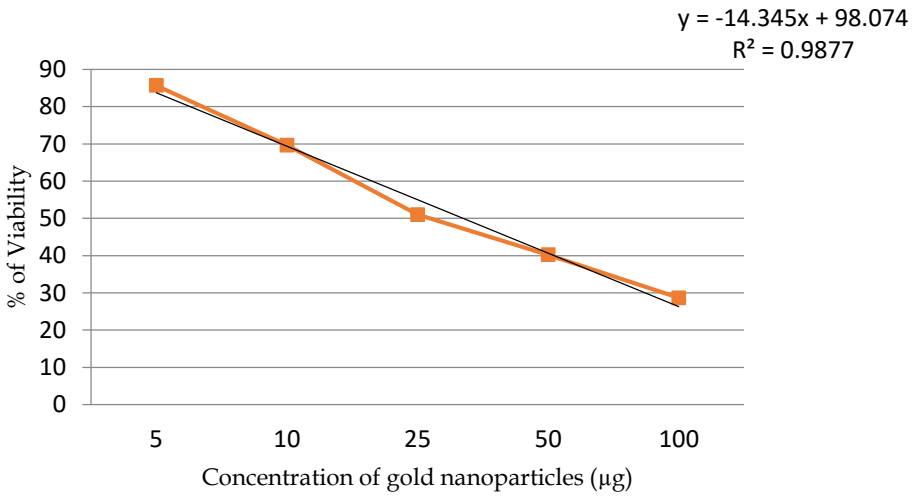

Figure-9. % of viability analysis of *Boswellia serrata* seed extract AuNPs on Normal fibro-blast cell line affected Drastically even at a high concentration of 100 mg/ml

pubescence was reported by Markus et al. [20]. They have attributed the antioxidant activity to the secondary metabolites like flavonoids, phenolic compounds and sesquiterpenes, present in *A.pubescens.*

3.6 *In Vitro* Cytotoxic Effect

The cytotoxic effect of green synthesized AuNPs was evaluated by MTT assay against human cervical cancer cell line (HeLa), human breast cancer cell line (MCF-7) and human normal cell line. **(**Figure.7, 8 and 9**)** shows the percent viability of HeLa, MCF-7 and normal fibroblast cells exposed to different concentrations (5, 10, 25, 50, 100µg/ml) of AuNPs. The % viability of HeLa cells, breast cells and normal cells were in the range of 84–31%, 97–28% and 85–28% when the concentration of synthesized AuNPs was in the range of 5–100µg/100µl, respectively. At low concentration of AuNPs, the % cell viability of HeLa cells, breast cells and normal cells was quite high but as concentration on AuNP increased, the % cell viability decreased.

The standard anti-cancer drug Doxorubicin Values (2µM) was used as positive control which showed 13, 28 and 16% cell viability for HeLa cells, breast cells and normal cells respectively at 400 mg/ml. The synthesized AuNPs showed decreased % cell viability against fibro-blast normal cells which indicates that AuNPs showed less cytotoxic effect to normal cells. At 100 mg/ml concentration AuNPs treated HeLa cells showed 31% cell viability; and breast cancer cells showed 28% cell viabilities while normal cells showed 28% cell viability respectively. When cancer cells are treated with nanoparticles, the cell morphological characteristics is changed or altered. Rajan et al. [21] reported morphological changes in the cells when treated with different concentration of AuNPs. Chandrasekaran et al. [22] reported that breast cancer cell streated with sliver nanoparticles showed morphological and sub-cultured before use. The microorganisms studied are clinically important ones causing several infections, food borne diseases, spoilages, skin infection and it is essential to overcome them through some active therapeutic agents changes such as, cell clumping, cell rupture, inhibition of cell growth and loss of

membrane stability. Nanoparticles also induce apoptosis of cell via several apoptotic mechanisms-like generations of ROS, activation caspase-3 cascade, and change apoptotic protein expression, cell cycle arrest which resulted in cell growth reduction, chromatin condensation, membrane blebbing and nuclear fragmentation [23, 24]. However, the cytotoxic effect of metal nanoparticles depends on size, shape, type of cells, capping agents and are also dose and time-dependent

4. Conclusion

We report a simple, easy, quick one-step green synthesis of AuNPs which is reproducible and ecofriendly which does not require any other reducing agent. The biomolecules in the aqueous seed extract of Boswellia Serrata act as reducing and stabilizing agent. AuNPs showed characteristic peak at 537 nm. The green synthesized AuNPs were predominantly round average size was 19.45 nm. Antimicrobial activity was done against a number of pathogenic microorganisms and green symthesized AuNPs exhibited antimicrobial activity more towards Gram-positive bacteria. They also showed dose-dependent DPPH, antioxidant activities and exhibited potential cytotoxicity on HeLa cancer cell line, MCF-7 cell line and Normal fibro-blast cell line. The results suggest that Boswellia Serrata seed extract can be effectively used for green synthesis of AuNPs that can be used as a natural antimicrobial, antioxidant and anticancer agent.. Boswellia Serrata mediated synthesized AuNPs could be employed as a source for the exploration of novel therapeutic agents in biomedical field to control various human diseases.

Conflicting Interests

The authors have declared that no conflicting interests exist.

References

[1] Chanda S, Amrutiya N, Rakholiya K. Evaluation of antioxidantproperties of some Indian vegetable and fruit peels by decoction extraction method. Am J Food Technol. 2013;8(3):173–182.

[2] Yemis GP,, Bach S, Delaquis P. Antibacterial activity of polyphenol-rich pomegranate peel extract against Cronobacter sakazakii. Int JFood Prop. 2019;22(1):985–993.

[3] Basri HH, Talib RA, Sukor R, et al. Effect of synthesis temperature on the size of ZnO nanoparticles derived from pineapple peel extract and antibacterial activity of ZnO–Starch nanocomposite films. Nanomaterials. 2020;10(6):1061.

[4] Donga S, Chanda S. Best from waste: therapeutic potential ofplant waste (seeds, peels, flowers). Int J Curr Microbiol Appl Sci.2020;9(08).

[5] Parvez GMM. Pharmacological activities of mango (Mangifera ind-ica): a review. J Pharmacogn Phytochem. 2016;5(3):1–7.

[6] Pande J, Chanda S. Screening of anticancer properties of somemedicinal plants–review. Int J Curr Microbiol Appl Sci. 2020;9(3):1348–1362.

[7] Mohanpuria P, Rana NK, Yadav SK. Biosynthesis of nanoparticles:technological concepts and future applications. J Nanopart Res.2008;10(3):507–517.

[8] Mukherjee S, Sau S, Madhuri D, et al. Green synthesis and charac-terization of monodispersed gold nanoparticles: toxicity study,delivery of doxorubicin and its bio-distribution in mouse model. JBiomed Nanotechnol. 2016;12(1):165–181.

[9] Milanezi FG, Meireles LM, de Christo Scherer MM, et al.Antioxidant, antimicrobial and cytotoxic activities of gold nano-particles capped with quercetin. Saudi Pharm J. 2019;27(7):968–974.

[10] Vijayan R, Joseph S, Mathew B. Anticancer, antimicrobial, antioxi-dant, and catalytic activities of green-synthesized silver and goldnanoparticles usingBauhinia purpurealeaf extract. BioprocessBiosyst Eng. 2019;42(2):305–319.

[11] Chahardoli A, Karimi N, Sadeghi F, et al. Green approach for syn-thesis of gold nanoparticles fromNigella arvensisleaf extract andevaluation of their antibacterial, antioxidant, cytotoxicity and cata-lytic activities. Artif Cells Nanomed Biotechnol. 2018;46(3):579–588.

[12] Perez C, Paul M, Bazerque P. An antibiotic assay by the agar well diffusion method. Acta Biol Med Exp. 1990; 15:113–115.

[13] Labieniec M, Gabryelak T. Effects of tannins on Chinese hamstercell line B14. Mutat Res Genet Toxicol Environ Mutagen. 2003;539(1–2):127–135.

[14] Nagalingam M, Kalpana VN, Rajeswari D, et al. Biosynthesis, char-acterization, and evaluation of bioactivities of leaf extract-medi-ated biocompatible gold nanoparticles fromAlternantherabettzickiana. Biotechnol Rep . 2018;19:e00268.

[15] Patra JK, Baek KH. Novel green synthesis of gold nanoparticlesusingCitrullus lanatusrind and investigation of proteasomeinhibitory activity, antibacterial, and antioxidant potential. Int JNanomed. 2015;10:7253–7264.

[16] K€onen-Adıg€uzel S, Adıg€uzel AO, Ay H, et al. Genotoxic, cytotoxic,antimicrobial and antioxidant properties of gold nanoparticlessynthesized byNocardiasp. GTS18 using response surface meth-odology. Mater Res Express. 2018;5(11):115402.

[17] Barai AC, Paul K, Dey A, et al. Green synthesis ofNerium oleander-conjugated gold nanoparticles and study of itsin vitroanticanceractivity on MCF-7 cell lines and catalytic activity. Nano Converg.2018;5(1):1–9.

[18] Singh P, Pandit S, Garnaes J, et al. Green synthesis of gold and sil-ver nanoparticles fromCannabis sativa(industrial hemp) and theircapacity for biofilm inhibition. Int J Nanomed. 2018;13:3571–3591.

[19] Gahlawat G, Choudhury AR. A review on the biosynthesis of metaland metal salt nanoparticles by microbes. RSC Adv. 2019;9(23):12944–12967.

[20] Markus J, Wang D, Kim YJ, et al. Biosynthesis, characterization,and bioactivities evaluation of silver and gold nanoparticles medi-ated by the roots of Chinese herbalAngelica pubescensMaxim.Nanoscale Res Lett. 2017;12(1):46.

[21] Rajan A, Vilas V, Philip D. Studies on catalytic, antioxidant, anti-bacterial and anticancer activities of biogenic gold nanoparticles.J Mol Liquid. 2015;212:331–339.

[22] Chandrasekaran R, Gnanasekar S, Seetharaman P, et al.Formulation ofCarica papayalatex-functionalized silver nanopar-ticles for its improved antibacterial and anticancer applications. JMol Liquid. 2016;219:232–238.

[23] Allen RT, Hunter WJ, Agrawal DK. Morphological and biochemicalcharacterization and analysis of apoptosis. J Pharmacol ToxicolMethod. 1997;37(4):215–228.

[24] Poojari, S., Porika, R., & Mamidala, E. (2014). Phytochemical analysis and in vitro antidiabetic activities of Physalis angulata fruit extracts. Natl. J. Integr. Res. Med, 5, 34-38.